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The Polymerase-Like Core of Brome Mosaic Virus 2a Protein, Lacking a Region Interacting with Viral 1a Protein In Vitro, Maintains Activity and 1a Selectivity in RNA Replication

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Brome mosaic virus (BMV), a member of the alphavirus-like superfamily of positive-strand RNA viruses, encodes two proteins required for viral RNA replication: 1a and 2a. 1a contains m^7G methyltransferase- and helicase-like domains, while 2a contains a polymerase (*pol*)-like core flanked by N- and C-terminal extensions. Genetic studies show that BMV RNA replication requires 1a-2a compatibility, implying direct or indirect 1a-2a interaction in vivo. In vitro, 1a interacts with the N-terminal 125-amino-acid segment of 2a preceding the *pol*-like core, and prior deletion studies suggested that this 2a segment was essential for RNA replication. We have now used protein fusions and deletions to explore possible parallels between noncovalent 1a-2a interaction and covalent fusion of similar protein domains in tobacco mosaic virus and to see whether the N-terminal 2a-1a interaction was the primary basis for 1a-2a compatibility in vivo. We found that 2a can function as part of a tobacco mosaic virus-like 1a-2a fusion and that a 2a segment (amino acids 162 to 697) comprising the *pol*-like core was sufficient to provide 2a functions in such a fusion. Unexpectedly, the unfused 2a core segment also supported RNA replication when it and wild-type 1a were expressed as separate proteins. Moreover, in gene reassortant experiments with the related cowpea chlorotic mottle virus, the unfused 2a core segment showed the same 1a compatibility requirements as did wild-type BMV 2a. Thus, the *pol*-like core of 2a must interact with 1a in a way that is selective and essential for RNA synthesis, and 1a-2a interactions are more complex than the single, previously mapped interaction of the N-terminal 2a segment with 1a.

The alphavirus-like superfamily encompasses a large number of animal and plant viruses that have 5′ capped, positive-strand RNA genomes and encode RNA replication proteins sharing sequence conservation in three domains (7, 15, 17). These three domains have similarities to m^7G methyltransferases (40), helicases (16), and RNA-dependent RNA polymerases (23), respectively. In different families within this superfamily these three domains are expressed as parts of one, two, or three proteins. For instance, the methyltransferase-, helicase-, and polymerase (*pol*)-like domains are present, respectively, in the nsP1, nsP2, and nsP4 proteins of alphaviruses (reviewed in reference 46) but are all part of a single 165-kDa polypeptide of potexviruses (18, 43). In part because of these varied coding strategies, one fundamental issue in understanding RNA replication by alphavirus-like viruses is whether and, if so, how interactions among these three conserved domains are involved in the assembly and/or function of the RNA replication complex.

One family of alphavirus-like viruses in which such interactions have begun to be identified are the bromoviruses. Bromovirus genomes consist of three RNAs. RNA3 encodes a host-specific, cell-to-cell movement protein (6, 9, 33) and the coat protein (2), while RNA1 and RNA2 encode all viral information required for RNA replication (26). RNA1 encodes 1a, a 109-kDa protein, containing methyltransferase- and helicase-like domains. RNA2 encodes 2a, a 94-kDa protein with a *pol*-like core flanked by N- and C-terminal segments of 200 and 125 amino acids (aa), respectively (Fig. 1A) (reviewed in reference 1). As discussed below, these N- and C-terminal 2a segments lack counterparts in many other alphavirus-like agents, such as tobacco mosaic virus (TMV).

Gene exchanges between the two best-studied bromoviruses, brome mosaic virus (BMV) and cowpea chlorotic mottle virus (CCMV), show that RNA replication requires compatibility between 1a and 2a (4, 12). Thus, at least at some steps, RNA replication must depend on 1a-2a interaction. Specifically, BMV RNA3 is replicated to high levels by expression of either BMV 1a and 2a or CCMV 1a and 2a but not by expression of either heterologous combination of 1a and 2a. CCMV 1a and BMV 2a fail to carry out any detectable RNA synthesis. Interestingly, however, BMV 1a and CCMV 2a support negative-strand RNA synthesis at 50% of the wild-type (wt) level, while positive-strand RNA3 synthesis is inhibited 30-fold (12). Thus, bromovirus positive- and negative-strand RNA syntheses appear to involve different aspects or modes of 1a-2a interaction. Similarly, recent work shows that for Sindbis virus, an animal alphavirus, termination of negative-strand synthesis and activation of efficient positive-strand synthesis are induced by protease cleavages that alter linkages between the conserved domains shared with BMV 1a and 2a (31). Studying the interactions among such RNA replication factors therefore appears important for understanding not only replication complex assembly but also the distinct mechanisms and regulation of positive- and negative-strand RNA synthesis.

Complementing the above-described genetic experiments, biochemical studies with BMV show that sequences in the N-terminal segment preceding the *pol*-like core of 2a interact in vitro with the helicase-like region of 1a (24, 25). Consistent with a functional role for this interaction, previous studies showed that some deletions in the RNA2 region coding for

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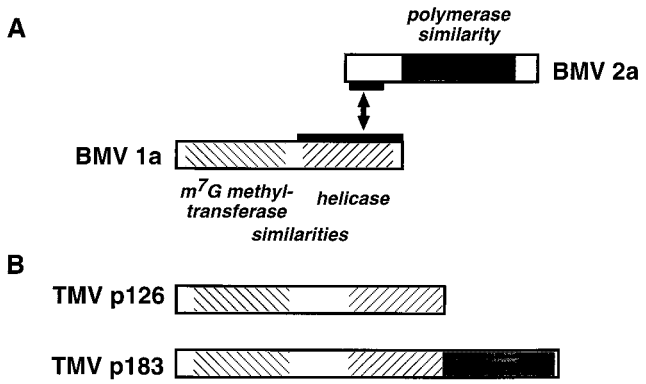

FIG. 1. Similarities between BMV (A) and TMV (B) RNA replication proteins. The hatched boxes indicate the intervirally conserved m^7G methyltransferase-like and helicase-like domains of BMV 1a (approximately aa 20 to 405 and aa 510 to 962, respectively) and the TMV 126-kDa protein (3). The shaded boxes correspond to the conserved *pol*-like domains of BMV 2a (approximately aa 203 to 685) and the TMV 183-kDa protein (17). The sequences previously found to direct an in vitro interaction between the BMV 1a helicase-like domain and the N-terminal region of 2a are overlined and underlined, respectively, in panel A.

these N-terminal 2a sequences can interfere with BMV RNA replication (50). Moreover, some aspects of this 1a-2a interaction appear to have parallels with the association of related protein domains in a TMV RNA replication factor, the 183-kDa readthrough protein (Fig. 1B). The 5′ end of the TMV genome encodes a 126-kDa protein similar to BMV 1a, followed by a leaky termination codon and the coding region for a *pol*-like domain related to the central portion of BMV 2a. Occasional (5 to 10%) translational readthrough of the leaky termination codon produces a protein of 183 kDa (reviewed in reference 8). Thus, in the 183-kDa protein the N terminus of the TMV *pol*-like domain is fused covalently to the helicase-like domain of 126 kDa, while for BMV the extended N terminus of the *pol*-like 2a interacts noncovalently with the helicase-like region of 1a (Fig. 1).

Together, the results described in the preceding two paragraphs suggested that the observed noncovalent interaction between 1a and the N-terminal region of 2a might be the basis for the compatibility requirement between 1a and 2a and that the resultant noncovalent 1a-2a complex might be a functional analog of the direct covalent fusion of methyltransferase-, helicase-, and *pol*-like domains in the TMV 183-kDa protein. Here we report further tests of these hypotheses using 183-kDa-protein-like 1a-2a fusions and 2a deletion mutants. The results reveal new aspects of 2a protein function and the interaction of 2a with 1a in RNA replication.

MATERIALS AND METHODS

Site-specific mutagenesis and original construction of fusion genes. Oligonucleotide-directed mutagenesis was performed on dUTP-single-stranded DNA (ssDNA) (30) or by PCR (41). Desired mutations were confirmed by restriction analysis and/or sequencing. The absence of unwanted mutations was assured by transferring a sequenced portion of the mutagenized fragment into its original context.

To facilitate construction of 1a-2a gene fusions, *Bam*HI sites were introduced into pB1TP3 and pB2TP5, plasmids coding for wt BMV RNA1 and RNA2, respectively (22) (Fig. 2). The primer d(ACAAGCGCATCACTGGATCCCCT TAACACAATT) was used to insert a *Bam*HI site (doubly underlined) 5′ to the 1a gene termination codon (RNA1 bases 2958 to 2960, complementary sequence underlined) in pB1TP3, giving pB1NL2 (1a-BamHI in Fig. 2). The primer d(C GAAGACATCTGGATCCTAGTAGAAAG) was used to create a *Bam*HI site (doubly underlined) upstream of the 2a gene initiation codon (RNA2 bases 104 to 106, complementary primer sequence underlined) of pB2TP5, giving pB2NL2 (2a-BamHI in Fig. 2). pF2, a plasmid carrying the direct fusion of the 2a gene to the 3′ end of the 1a gene, was obtained by transferring the *Bam*HI-*Eco*RI fragment of pB2NL2 (RNA2 bases 96 to 2867) to *Bam*HI-, *Eco*RI-cut pB1NL2.

The second fusion constructed contained the BMV 1a sequence from pB1NL1 and BMV 2a codons 163 to 697 separated by a 33-nucleotide-long sequence from TMV (bases 3402 to 3435) (14), which contains the leaky amber terminator of the TMV 126-kDa gene with five adjacent TMV codons on each side (44, 45). First, an oligonucleotide containing the relevant TMV sequence (bold, with the leaky codon italicized) flanked on the 5′ side by a *Bam*HI site (doubly underlined) and on the 3′ side by BMV 2a codons 162 to 168 (underlined), d(AGGGGATCCAG**GATGCAGGAACACAA*TAG*CAATTACAGATTGAC** GATAC$_5$AAGGAGCTGACC), was used for PCR mutagenesis of *Eco*RI-linearized pB2PT50. pB2PT50 contains a deletion variant of BMV RNA2 cDNA that encodes a 3′-truncated 2a gene consisting of 2a codons 1 to 697 followed by a single out-of-frame alanine codon and a TAA termination codon (50). Immediately 3′ to this termination codon, a second *Bam*HI site (doubly underlined) was introduced with d(AGGGGGATCCTTAGGCACAGTAGCAATCAGA GAA), which served as another primer in the same reaction. The *Bam*HI fragment of the PCR product was inserted into *Bam*HI-cut pB1NL2. The resulting plasmid, pF21, thus contained an entire BMV 1a gene fused at its 3′ end to a *Bam*HI site, the TMV leaky terminator region, and a 5′,3′-truncated portion of the BMV 2a gene. To facilitate further manipulations, pF25 was obtained by transferring the entire fusion gene (a *Pst*I-*Eco*RI fragment) from pF21 into *Pst*I-, *Eco*RI-cut pUC118. Thus, pF25 contained all viral sequences present in pF21 but in a different orientation in the vector.

The leaky termination codon (TAG) in the TMV-derived portion of pF25 dUTP-ssDNA was mutated to a sense codon (TAT, bold) with d(GCAGGAA CACAA**TAT**CAATTACAGATT), giving pF26. The entire TMV sequence was removed from the 1a-2a fusion gene by site-specific mutagenesis of pF21 with d(AG$_2$GGATCCAGGATAC$_5$AAGGAGCTGACC) to give pF11 (*Bam*HI site doubly underlined; BMV 2a codons 162 to 168 underlined).

Plasmids for transient expression in vivo. All plasmids for transient expression of viral proteins in plant protoplasts were based on pRT101, a plasmid that carries the cauliflower mosaic virus 35S promoter and polyadenylation signal (48). Where appropriate, the laboratory designation of each plasmid is given in parentheses following the short descriptive name used in this paper. pB1 (pB1PA17) and pB2 (pB2PA17) contain the coding sequences of BMV 1a and 2a genes, respectively, in pRT101 and were described by Dinant et al. (12). pC1 (pCC1SD3) and pC2 (pCC2SD3) carry, respectively, the 1a and 2a genes of CCMV in pRT101 (12).

Plasmids for transient expression of selected BMV 1a insertion mutants (see Results) were constructed from pB1 by replacing the unique internal *Cla*I fragment (RNA1 bases 87 to 873) or *Sal*I fragment (RNA1 bases 1178 to 2831) of the 1a gene with the corresponding *Cla*I fragment from pB1PK6, pB1PK7, or pB1PK10 (29) or *Sal*I fragment from pB1PK15 (29).

p2aΔC (pB2KS15) contained a C-terminally truncated BMV 2a gene (aa 1 to 697 of wt 2a) in pRT101. This 2a fragment was identical to the one in pB2TP50 (50) but was flanked on its 3′ side by a *Bam*HI site. Thus, the C terminus of 2aΔC was identical to the C terminus of all 1a-2a fusions tested in this study (Fig. 2). p2aΔC was constructed as follows. The *Nco*I-*Bam*HI fragment of pF21 (encoding 2a aa 261 to 697 followed by a stop codon; see above) was used to replace the *Nco*I-*Stu*I fragment of pB2TP5 (RNA2 bases 882 to 2500). The *Fsp*I-*Hin*dIII fragment (RNA2 bases 44 to 2293) of this intermediate plasmid was made blunt ended with the Klenow fragment of DNA polymerase I and inserted into the pRT101 *Sma*I site (position 694).

p1a2aΔNC (pB1KS17) was constructed by transferring the *Sac*II-*Xho*I fragment of 1a-2a fusion pF11 (the *Sac*II site corresponds to position 2284 of BMV RNA1, while *Xho*I cuts 3′ to the 2a sequence in the fusion) to *Sac*II-, *Xho*I-cut pB1. p1a*2aΔNC (pB1KS18) and p1a~2aΔNC (pB1KS19) were constructed similarly by transferring the *Sac*II-*Xho*I fragments of pF25 and pF26, respectively, to *Sac*II-, *Xho*I-cut pB1. p1a2aΔC (pB1KS20) was obtained by transferring the internal *Nco*I fragment of pF2 to *Nco*I-cut p1a~2aΔNC. *Nco*I sites are unique in the BMV 1a and 2a coding sequences (positions 2116 and 882, respectively).

Deletion mutants of p1a2aΔNC (see Fig. 4A) were constructed as follows. pΔI (pB1KS29) and pΔII (pB1KS30) were obtained by deleting the unique internal *Cla*I fragment (1a codons 6 to 267) and *Sal*I fragment (1a codons 370 to 919), respectively, from p1a2aΔNC. Double deletion mutant pΔIII (pB1KS33) was derived from pΔI by deleting the *Sal*I fragment (codons 370 to 919) identical to the one missing in pΔII. pΔIV (pB1KS32) was obtained by deleting the *Cla*I-*Sma*I fragment (1a codons 267 to 296) from pΔIII. pΔV (pB1KS31) was constructed as follows (see Fig. 4A). pΔIII was fully digested with *Cla*I and partially digested with *Sca*I (to cleave at the site corresponding to position 2863 in RNA1). The restriction products were separated on an agarose gel. The band of the desired size was eluted and treated with the Klenow fragment of DNA polymerase I to fill in the ends prior to ligation. Thus, the only wt 1a amino acids encoded in pΔV were the first 6 N-terminal and the last 30 C-terminal residues. All p1a2aΔNC deletion mutants described above preserved the 1a reading frame.

p2aΔNC (pB2KS35) was constructed to transiently express the central portion of 2a (aa 162 to 697) contained in 1a2aΔNC (Fig. 2) and its deletion derivatives (see Fig. 4). This 2a region was PCR amplified from pΔV. One PCR primer, d(CCCAAGCTT**ATG**GGATCCAGGATAC$_5$AAGG), preserved the *Bam*HI site (doubly underlined) 5′ to the start of the 2a sequence in p1a2aΔNC and pΔV, etc., and introduced an in-frame initiation codon (bold) and a flanking

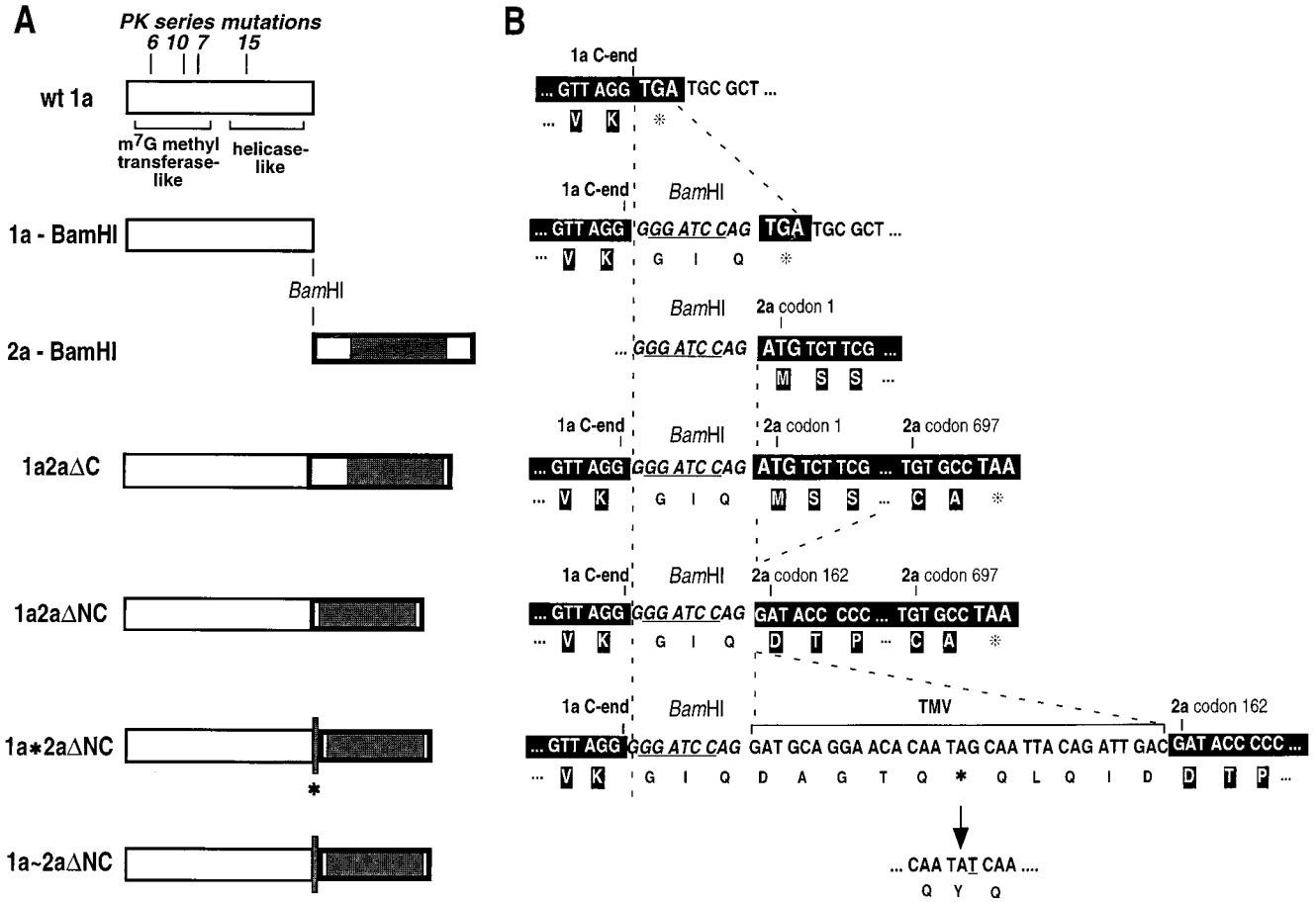

FIG. 2. (A) Schematic map of BMV-derived 1a and 2a ORFs and their fusions. The locations of PK series mutations (see Results) are indicated above the box corresponding to the wt 1a ORF. The shaded box corresponds to the *pol*-like core of the 2a protein. The 2a portion of all 1a-2a fusions carries a 3′ deletion identical to that of the previously characterized PT50 mutant (50). The 2a-derived region present in 1a2aΔNC, 1a*2aΔNC, and 1a~2aΔNC corresponds to aa 162 to 697 of wt 2a. The shaded bar between the 1a and 2a sequences in 1a*2aΔNC and 1a~2aΔNC corresponds to 33 nt from TMV genomic RNA, surrounding the leaky termination codon for TMV 126-kDa protein (Fig. 1B and Results). The asterisk denotes the TMV leaky termination codon in 1a*2aΔNC. This amber codon is mutated to a sense codon (TAG→TAT) in 1a~2aΔNC (see panel B). (B) Sequences of the modified ends of the BMV 1a and 2a genes and the 1a-2a fusion junctions of the constructs shown in panel A. The *Bam*HI sites introduced to facilitate 1a-2a fusion are underlined. BMV 1a- and 2a-derived coding regions are highlighted against a dark background. The amino acids encoded are indicated below the corresponding RNA sequences.

*Hin*dIII site (underlined) 5′ to this *Bam*HI site. The second PCR primer, d(GG GAAGCTTGGATCCTTAGGCACAGTAGCAATCAGAG), preserved the *Bam*HI site (doubly underlined) 3′ to the end of the 2a sequence in p1a2aΔNC and pΔV and introduced a *Hin*dIII site (underlined) immediately 3′ to this *Bam*HI site. The resulting PCR fragment was cloned in the pCNTR shuttle vector by using the General Contractor DNA cloning system (5 Prime→3 Prime, Inc., Boulder, Colo.). The *Hin*dIII sites introduced by PCR were used to cleave out the fragment of interest, which was ligated into the pRT101 *Bam*HI site (position 699) after both fragment and vector were treated with the Klenow fragment of DNA polymerase I. Since this does not regenerate a *Bam*HI site at the ligation junction, the resulting plasmid contained the N- and C-terminally truncated 2a sequence flanked by the ATG and the two *Bam*HI sites generated by PCR. To ensure that no unwanted mutations had been introduced by PCR, the *Bam*HI-flanked 2a segment was replaced by its counterpart from pΔV. Consequently, the resulting final plasmid p2aΔNC contained an initiation codon upstream of the truncated 2a gene sequence (codons 162 to 697) present in p1a2aΔNC and its deletion derivatives but, unlike these, lacked any 1a amino acid residues.

The structures of all plasmids were confirmed by multiple restriction digests and, in some instances, sequencing.

In vitro transcription. In vitro transcription reactions were performed as described elsewhere (28). T7 RNA polymerase transcripts of wt BMV RNA3 were synthesized from *Eco*RI-linearized pB3TP8 (22). Transcripts of B3CAT, a BMV RNA3 derivative in which the coat protein gene has been replaced by the chloramphenicol acetyltransferase (CAT) gene, were synthesized from *Eco*RI-cleaved pB3CA101 (21).

Transfection of *Nicotiana benthamiana* protoplasts. *N. benthamiana* protoplasts were isolated essentially as described elsewhere (12), with the following modifications. After overnight incubation of leaves in an enzyme solution (0.5% macerozyme, 2% cellulase), viable protoplasts were separated from cell debris by filtration, followed by centrifugation onto a 20% sucrose cushion. The protoplasts were then transferred to a fresh tube, washed twice with 10% mannitol, and subjected to a mixed RNA-DNA transfection with polyethylene glycol-$CaCl_2$ as described previously (12, 28). Protoplasts were harvested 24 h postinoculation (hpi) for RNA analysis and 30 hpi for CAT assay.

RNA analysis and CAT assay. Northern (RNA) blot analysis of viral RNA and a CAT assay were performed as previously described (12, 28). Prior to autoradiography, the membranes (Northern blot) and thin-layer chromatography plates (CAT analysis) were scanned by using a PhosphorImager model 425 system (Molecular Dynamics, Sunnyvale, Calif.).

RESULTS

One goal of this work was to explore the possible relation between the in vitro interaction of the BMV 2a N-terminal region with the 1a C-terminal region (Fig. 1A) (24, 25) and the in vivo requirement for 1a-2a compatibility for RNA replication (4, 12). To this end, following the parallels outlined in the introduction, we first constructed a series of 1a-2a fusions modeled after the TMV 183-kDa protein (Fig. 1B). These fusions were then tested in protoplasts to determine whether the known noncovalent 1a-2a interaction could be replaced, and the N-terminal 2a segment involved in it made dispens-

able, by 183-kDa-protein-like covalent linkage of 2a sequences to the 1a C terminus. For expression, each relevant BMV-derived open reading frame (ORF) was inserted in a plasmid between the 35S promoter and the polyadenylation signal of cauliflower mosaic virus (47). Expression of wt 1a and 2a from such plasmids supports BMV RNA3 replication and subgenomic mRNA transcription (see below) at levels equal to or above those for wt BMV infection (12). To test the 1a and 2a variants used in this study, *N. benthamiana* protoplasts were cotransfected with the appropriate 35S expression plasmids and in vitro transcripts of wt BMV RNA3. All fusions and other derivatives were tested in multiple independent transfection experiments, and representative results are shown in the figures.

Fusions of truncated 2a variants to 1a support RNA replication only with wt 1a. The C-terminal 125 aa of 2a are dispensable for RNA replication in protoplasts (50). Accordingly, we tested fusion 1a2aΔC (Fig. 2), in which 2a with the previously characterized 125-aa C-terminal truncation was joined to the 1a C terminus, duplicating the methyltransferase-, helicase-, and *pol*-like order of the TMV 183-kDa protein (Fig. 1B). Neither positive- nor negative-strand RNA synthesis was detected after protoplasts were inoculated with wt RNA3 transcripts and a plasmid expressing 1a2aΔC alone (Fig. 3A and B, lanes 2). Addition to the inoculum of pB2, a plasmid expressing wt 2a, did not lead to detectable RNA synthesis (Fig. 3A and B, lanes 10). However, when coinoculated with pB1, a plasmid expressing wt BMV 1a, the fusion supported negative- and positive-strand RNA3 synthesis to approximately 30% of wt levels (Fig. 3A and B, lanes 6) as quantified with a PhosphorImager system (see Materials and Methods).

We next deleted the first 161 aa from the 2a portion of 1a2aΔC to obtain 1a2aΔNC (Fig. 2). This deletion included the entire region (aa 40 to 125) previously found to be necessary and sufficient for interaction with 1a in vitro (24). To further enhance the similarity to TMV, we also constructed p1a*2aΔNC (Fig. 2) by inserting 33 nt of TMV RNA sequence, surrounding the leaky 126-kDa ORF amber termination codon, between the 1a and 2a portions of p1a2aΔNC (Fig. 2). Since this TMV segment directs translational readthrough at a frequency of approximately 5% (44), 1a*2aΔNC mRNA translation should produce both unfused 1a and the 1a*2aΔNC fusion protein from a single ORF, in direct parallel to the situation with TMV. As a control for other possible effects of the TMV sequence, the amber terminator (TAG) in p1a*2aΔNC was mutated to a tyrosine codon (TAT), giving p1a~2aΔNC (Fig. 2).

In the presence of 1a coexpressed from pB1, all three fusions lacking the first 161 aa of 2a supported RNA3 replication (Fig. 3A and B, lanes 7 to 9). There was no consistent difference among these three fusions or between the fusions and 1a2aΔC in negative-strand RNA3 accumulation (Fig. 3A, lanes 6 to 9; see also lane 4). However, for 1a*2aΔNC and 1a~2aΔNC, the level of positive-strand RNA3 was typically 10 to 20% that of wt 2a (Fig. 3B, lane 8 and 9), while that for 1a2aΔNC was lower and more variable, ranging in different experiments from approximately 1% (Fig. 3B, lane 7) to 10% (Fig. 4C, lane 1, and other experiments) that of wt 2a.

Like 1a2aΔC, RNA3 replication by 1a2aΔNC and 1a~2aΔNC remained strictly dependent on 1a coexpression from the separate pB1 plasmid (Fig. 3A and B, lanes 3 and 5 versus 7 and 9). However, as intended, the TMV leaky termination codon fragment inserted in 1a*2aΔNC to provide self-expression of unfused 1a allowed it to support RNA3 replication without pB1 (Fig. 3A and B, lane 4). This independent activity of 1a*2aΔNC was due to the leaky termination codon following 1a, since 1a*2aΔNC and 1a~2aΔNC differed only by a single base in that codon (Fig. 2 and above). Consistent with this, coinoculation of pB1 with p1a*2aΔNC did not increase RNA3 accumulation (Fig. 3A and B, lanes 8). Furthermore, while coexpression of wt 2a from pB2 had no effect on the other fusions, it increased positive-strand RNA3 accumulation with 1a*2aΔNC to nearly wt levels, presumably because of coexpression of wt 1a (from p1a*2aΔNC) and wt 2a (from pB2) (Fig. 3A and B, lanes 10 to 13).

For each of the fusions, accumulation of RNA3 was also accompanied by a band of the expected size for BMV RNA4, the subgenomic coat protein mRNA synthesized by internal initiation on negative-strand RNA3 (Fig. 3B, lanes 4 and 6

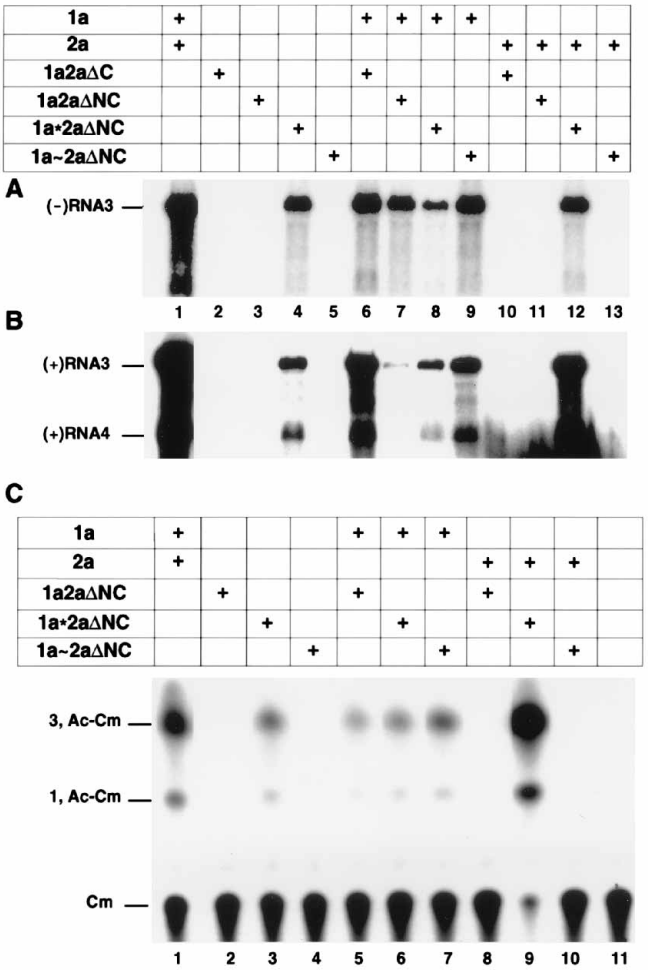

FIG. 3. Representative RNA replication and transcription assays with 1a-2a fusions. *N. benthamiana* protoplasts were inoculated with plasmids expressing the indicated combinations of wt 1a, 2a, and their fusions, together with in vitro transcripts of either wt RNA3 (A and B) or its derivative B3CAT, in which the coat protein gene was replaced by the CAT gene (C). Protoplasts were harvested 24 hpi for RNA analysis and 30 hpi for CAT assay. (A and B) Northern blot analysis of negative-strand (A) and positive-strand (B) RNA accumulation. Upon protoplast harvesting, total RNA was extracted, glyoxylated, and electrophoresed in a 1% agarose gel. After transfer to a nylon membrane (Hybond; Amersham) and prehybridization, the membranes were probed with ^{32}P-labeled transcripts corresponding to the 200-nt-long 3′ nontranslated region conserved in all BMV RNAs to detect negative-strand RNA3 accumulation (A) or with transcripts complementary to the same sequence to detect positive-strand RNA accumulation (B). The lane for wt 1a and 2a (lane 1) on the membrane was intentionally overexposed to better visualize RNA in other lanes for comparative purposes. The positions of viral RNAs are shown on the left. (C) CAT assays of extracts from protoplasts inoculated with B3CAT in vitro transcripts and plasmids expressing the combination of proteins indicated above each lane. The positions of the acetylated (Ac) forms of [^{14}C]chloramphenicol (Cm) are indicated on the left. The inoculum in lane 11 contained only water.

to 9; for 1a2aΔNC, see also Fig. 4C, lane 1). To confirm that these bands represented active subgenomic mRNA transcripts rather than RNA3 degradation products, protoplasts were transfected with plasmids expressing appropriate combinations of 1a, 2a, and their fusion proteins together with in vitro transcripts of RNA3 derivative B3CAT, in which the coat protein gene was replaced by CAT. At 30 hpi, extracts of these cells were tested for CAT activity, which as noted above requires BMV-directed negative-strand synthesis and transcription of RNA4 (13, 21). As shown in Fig. 3C, these assays confirmed the Northern results, showing that all of the fusions supported CAT expression and, thus, subgenomic mRNA transcription, but only in the presence of wt 1a, expressed either from pB1 or, for p1a*2aΔNC, from the fusion gene itself.

Effect of 1a mutations on RNA replication by 1a plus 1a2aΔNC. The above fusion results showed that the N-terminal 2a sequences that interact with 1a were not essential for RNA3 replication and transcription in the context of a TMV 183-kDa-protein-like 1a-2a fusion. Moreover, mimicking TMV gene organization in p1a*2aΔNC allowed expression of a functional, BMV-derived RNA replication apparatus from a single RNA. However, the ability of the 1a-2a fusions to support RNA synthesis only upon coexpression of unfused 1a and their inability to direct RNA synthesis when coexpressed with wt 2a showed that the 1a portion of the fusion was unable to provide at least some functions of unfused 1a. This dependence of 1a-2a fusions on unfused 1a paralleled wt TMV expression of the 1a-like 126-kDa protein as well as the 183-kDa protein (see Discussion), but the relative contributions of 1a sequences in the unfused and 2a-fused forms remained unclear.

To examine the contributions of various sequences in unfused 1a, four 2-amino-acid insertion mutations, previously shown to abolish the ability of 1a to support RNA replication with wt 2a (29), were transferred individually to pB1. As shown at the top of Fig. 2A, three of these mutations, PK6, PK7, and PK10, are located in the m^7G methyltransferase-like domain, while one, PK15, is located in the helicase-like domain (29). When protoplast samples were transfected with each of these four mutant 1a expression plasmids in turn, together with the 1a2aΔNC expression plasmid and wt RNA3 or B3CAT in vitro transcripts, neither positive- nor negative-strand RNA3 synthesis nor CAT expression was detected (results not shown). Thus, the wt 1a portion of 1a2aΔNC failed to complement any of these mutations in unfused 1a, suggesting that the expression of both 1a domains as parts of unfused 1a was required for RNA replication.

The 2a *pol*-like core functions without fusion to 1a. To examine the contributions of different 1a segments within 1a2aΔNC, a series of in-frame deletions in the 1a portion of this fusion gene was created and the resulting expression plasmids were tested in protoplasts cotransfected with wt pB1 and wt RNA3 transcripts. First we deleted nonoverlapping N-proximal *Cla*I and C-proximal *Sal*I fragments to create ΔI and ΔII, respectively (Fig. 4A). Although ΔII lacked nearly all of the helicase-like domain and the C-terminal part of the m^7G methyltransferase-like domain, it supported full RNA3 replication, with negative-strand accumulation reduced relative to that of 1a2aΔNC but positive-strand RNA3 and RNA4 accumulation equal to the highest levels supported by 1a2aΔNC (Fig. 4B and C, lanes 1 and 3). Thus, neither 1a domain was required intact for the 1a-2a fusion to function. For ΔI, which lacked the N-proximal half of the methyltransferase-like region, negative- and positive-strand RNA3 accumulations were severely reduced (Fig. 4B and C, lanes 2). However, this behavior was specific to ΔI; ΔIII, ΔIV, and ΔV showed that deletion of the same N-proximal region together with progressively larger

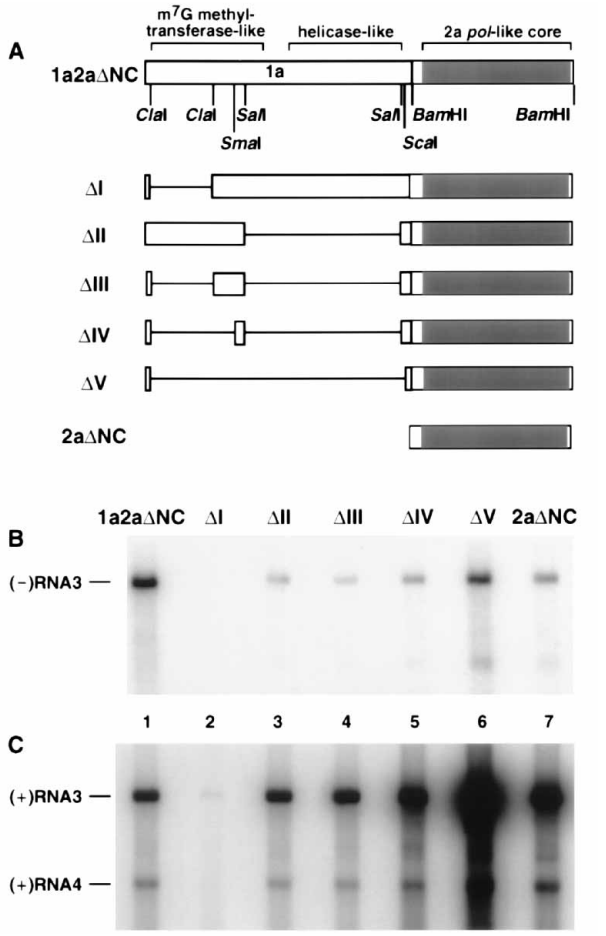

FIG. 4. Representative RNA replication assays with 1a2aΔNC deletion mutants. (A) Schematic representation of 1a2aΔNC and its deletion derivatives. The plain boxes correspond to the 1a portion of the fusion or remaining parts thereof. The restriction enzyme sites used to engineer the deletions are shown below the 1a portion of 1a2aΔNC (see Materials and Methods for construction details and the specific 1a codons deleted in each case). Single horizontal lines correspond to the deleted portions of 1a. The shaded box indicates the *pol*-like core of 2a. (B and C) Northern blot analysis of negative-strand (B) and positive-strand (C) RNA accumulation in plant protoplasts. *N. benthamiana* protoplasts were inoculated with in vitro transcripts of wt RNA3, a plasmid expressing wt 1a, and a second plasmid expressing the 1a2aΔNC derivative indicated above each lane. Total RNA extracted from protoplasts was subjected to Northern blotting as described in the legend to Fig. 3 and the corresponding section of Materials and Methods. The positions of viral RNAs are indicated on the left. A faint band corresponding to RNA3 can be seen in lanes 2 of panels B and C after longer exposures.

downstream portions of 1a supported increasing levels of RNA3 replication (Fig. 4B and C, lanes 4 to 6). For ΔV, in which nearly all the 1a sequence was deleted, positive-strand accumulation throughout five independent experiments equaled and sometimes even slightly exceeded that of wt 2a. For all deletions, subgenomic RNA4 production paralleled RNA3 replication (Fig. 4C; also, B3CAT results [not shown]).

Finally, oligonucleotide-directed mutagenesis was used to remove the first 4 aa and the last 30 aa of wt 1a from the N terminus of ΔV, yielding 2aΔNC (Fig. 4A). 2aΔNC also supported RNA3 replication without either the 1a-interactive N-terminal segment of wt 2a or direct fusion to any 1a sequence (Fig. 4B and C, lanes 7). These results were unexpected since, as noted in the introduction, previous attempts to identify functional 2a mutants with extensive N-terminal deletions were unsuccessful (50). Possible reasons for this prior lack of suc-

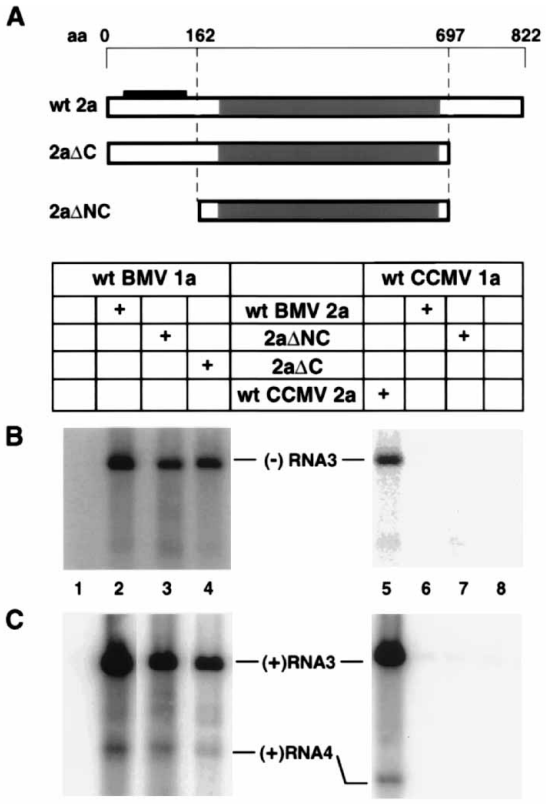

FIG. 5. 1a specificity in 2aΔNC function. (A) Diagram of wt BMV 2a protein and its deletion mutants 2aΔC (aa 1 to 697) and 2aΔNC (aa 162 to 697). The scale at the top shows amino acids in wt 2a. The shaded boxes correspond to the *pol*-like core of 2a. The bar above the wt 2a diagram indicates the N-terminal region required for interaction in vitro with the helicase-like domain of BMV 1a (24). (B and C) Representative Northern blot analysis of negative-strand (B) and positive-strand (C) viral RNA accumulation directed in vivo by the indicated combinations of BMV and CCMV 1a and 2a protein derivatives. *N. benthamiana* protoplasts were inoculated with in vitro transcripts of BMV RNA3 together with plasmids expressing wt BMV 1a (left) or CCMV 1a protein (right) and, as indicated above each lane, either no additional plasmid (lanes 1 and 8) or plasmids expressing wt BMV 2a, 2aΔNC, 2aΔC, or CCMV 2a. The positions of viral RNAs are shown in the center. Note that the faint positive-strand RNA3 bands in panel C, lanes 6 and 7, correspond to residual input RNA3 inoculum (lane 8).

cess, including the use of replication-dependent RNA2 derivatives and known *cis*-acting effects of the relevant deletions on RNA2 replication, are considered in the Discussion section.

2aΔNC retains high-level activity and 1a selectivity in RNA replication. To determine how loss of the N-terminal, 1a-interactive segment affected 2a function, we directly compared RNA replication levels in protoplasts transfected with pB1, wt RNA3 transcripts, and the expression plasmid for either 2aΔNC, wt 2a, or 2aΔC, which has the same C-terminal 125-aa deletion as 2aΔNC but retains the complete 2a N-terminal sequence (Fig. 5A). As shown by Traynor et al. (50), 2aΔC supported negative- and positive-strand RNA3 and RNA4 accumulation to approximately 75% of the level of wt 2a (Fig. 5B and C, lanes 4). For 2aΔNC, negative- and positive-strand RNA3 and RNA4 synthesis levels were only slightly lower, averaging 55% of the level of wt 2a over four experiments (Fig. 5B and C, lanes 3, and other experiments). Thus, loss of the N-terminal 2a sequences caused a relatively modest decline in activity for 2aΔNC relative to 2aΔC. As shown previously (12), no RNA synthesis was detected in inoculations with pB1 and wt RNA3 alone (Fig. 5B and C, lanes 1).

The ability of 2aΔNC to support RNA replication at levels approaching those of wt 2a provided the opportunity to meaningfully examine further the in vivo requirements for 1a-2a compatibility in RNA replication. As summarized in the introduction, exchanges of 1a and 2a between BMV and another bromovirus, CCMV, revealed that wt 1a and 2a operate interdependently (12), and in vitro interaction between BMV 1a and the N-terminal region of BMV 2a suggested that this might be the basis for 1a-2a compatibility (24). To ascertain whether this N-terminal region of wt 2a contained any crucial determinants for 1a-selective function, we tested whether 2aΔNC could support RNA replication with CCMV 1a. Protoplasts were transfected with BMV RNA3 and plasmids expressing wt CCMV 1a and either wt CCMV 2a, wt BMV 2a, or 2aΔNC (Fig. 5B and C, lanes 5 to 8). As found previously (12), CCMV 1a and 2a supported BMV RNA3 amplification and RNA4 transcription to levels similar to those obtained with BMV 1a and 2a, while CCMV 1a and wt BMV 2a supported no detectable positive- or negative-strand RNA synthesis (Fig. 5B and C, lanes 5 and 6). Most importantly, in repeated experiments, 2aΔNC duplicated the behavior of wt BMV 2a, also yielding no detectable positive- or negative-strand RNA synthesis when coexpressed with CCMV 1a (Fig. 5B and C, lanes 7). For both wt BMV 2a and 2aΔNC, close inspection demonstrated a faint hybridization signal for positive-strand RNA3, but the presence of an equivalent faint RNA3 band in transfections lacking any 2a expression plasmid showed that this represented residual, input RNA3 inoculum (Fig. 5C, lanes 6 to 8). Thus, like wt 2a, 2aΔNC selectively supported BMV RNA3 replication and transcription when coexpressed with BMV 1a but not when coexpressed with CCMV 1a, implying that the *pol*-like core of 2a contains sequences directing some form of 1a-2a interaction that is required for RNA synthesis in vivo.

DISCUSSION

While exploring the relation between an N-terminal 2a-1a interaction detected in vitro and BMV RNA replication in vivo, we found that the *pol*-like core of 2a was sufficient to support RNA replication, either as a part of a TMV-like fusion or, even more efficiently, when expressed as a separate protein with wt BMV 1a. As noted below, these results indicate that the *pol*-like core of 2a must interact with 1a in a specific fashion and have implications for the function of related protein domains encoded in free and fused form by other alphavirus-like viruses.

1a-2a fusions and their derivatives. BMV 1a-2a fusions modeled after the TMV 183-kDa protein were able to contribute to RNA replication, and, by using the TMV leaky terminator, it was possible to express a complete, functional BMV RNA replication apparatus from a single RNA. Although the fusions were designed to mimic TMV, the relation between the function of these 1a-2a fusions and the natural function of TMV replication factors is not yet clear. RNA replication with BMV 1a-2a fusions was dependent on coexpression of unfused 1a. By contrast, mutating the leaky UAG codon of the 126-kDa ORF to a sense codon suggests that 183-kDa protein may be sufficient for TMV RNA replication (19, 20), although the spontaneous reversion of such mutants to a termination codon implies that 126-kDa protein is required for optimal replication (19). Even if 183-kDa protein can direct RNA replication without free 126-kDa protein, the ability of 1a and 2aΔNC to support RNA replication (Fig. 5) suggests that fusion of the *pol*-like domain to 126-kDa protein might not be essential for TMV function but rather, as for 1a*2aΔNC, may be convenient for expressing multiple functions from a single RNA.

The presence of the full 1a sequence in 1a2aΔNC enhanced negative-strand RNA3 accumulation relative to levels for

ΔII-ΔV and 2aΔNC (Fig. 4B). This might result from general effects on protein folding or from interaction of 1a and 2a sequences within the fusion, or it may occur because possible 1a-1a interactions provided an alternate route for intermolecular interaction of the 2a sequences with a separate, unfused 1a. Conversely, deletion of N-terminal 2a sequences from 1a2aΔNC inhibited positive-strand synthesis more than negative-strand synthesis (Fig. 3A and B). Both of these effects are consistent with prior data indicating that BMV negative- and positive-strand syntheses have different requirements for 1a-2a interaction (12).

ΔV, comprising the 2a core fused to a few N-terminal 1a amino acids, was more active than 2aΔNC, which lacked any 1a residues (Fig. 4, lanes 6 and 7). We cannot say whether this represents a specific property of these 1a residues or some more general effect. Similarly, 1a*2aΔNC and 1a~2aΔNC were more active than 1a2aΔNC. Since these differed by insertion of heterologous TMV sequences between 1a and 2a (Fig. 2), the increased activity may have been due to general effects such as enhanced flexibility between 1a and 2a sequences due to the additional spacer residues.

Activity and 1a selectivity of 2aΔNC, a minimal 2a derivative. The high level of RNA accumulation supported by 2aΔNC indicates that, although shown to direct a specific interaction with the helicase-like domain of 1a (24, 34), the N-terminal 161 aa of 2a are not essential for RNA3 replication and transcription in protoplasts. Since 2aΔNC also lacked the C-terminal 125 aa of 2a, which were previously found to be nonessential for RNA replication (50), the N- and C-terminal extensions of the 2a *pol*-like core are simultaneously dispensable for BMV RNA synthesis in protoplasts.

Like wt 2a, 2aΔNC was able to function with BMV 1a but not CCMV 1a (Fig. 5). This 1a selectivity shows that 2aΔNC must carry sequences controlling a direct or indirect 1a-2a interaction required for RNA synthesis. Thus, at least two separate regions of wt 2a, the N-terminal segment and the central core, must be capable of interaction with 1a. The possibility that 1a-2a interaction may involve multiple interaction sites or other nuances was not unexpected in light of the complexity of RNA-dependent RNA polymerase functions, including initiation and elongation of RNA synthesis, differential regulation of negative- and positive-strand synthesis, and subgenomic mRNA transcription, etc. In particular, recent results showing that BMV negative- and positive-strand syntheses involve genetically distinguishable aspects of 1a-2a interaction (see reference 12 and above) and that protease cleavages between 1a- and 2a-homologous domains enhance alphavirus positive-strand synthesis at the expense of negative-strand synthesis (31, 42) suggest that 1a, 2a, and their homologs may have two or more alternate states of association that are important for different steps of replication.

Interaction of the 2a core with 1a may not have been revealed in prior in vitro tests of 1a-2a interaction (24, 25) for several reasons. This interaction might depend on specific conditions, such as the membrane environment in which 1a and 2a associate in vivo (36, 37). Alternatively, unlike the direct in vitro interaction between the 2a N-terminal region and the helicase-like domain (24, 25), interaction of the 1a and the *pol*-like core might require an additional intermediary factor or factors not present in prior in vitro tests.

The ability of 2aΔNC to support RNA replication was surprising because in a prior study, small deletions in the RNA2 region coding for the N-terminal segment of 2a reduced RNA synthesis and deletions of 2a codons 2 to 102 or 2 to 203 abolished detectable RNA replication (50). Several factors may explain why these earlier RNA2 mutants did not support detectable RNA synthesis. First, even minor alterations in deletion endpoints can dramatically alter protein activity, possibly because of effects on protein conformation or stability. Prior analysis of the BMV 2a C-terminal region, e.g., showed that while the 125-aa deletion variant 2aΔC retains 75% of wt 2a activity (Fig. 5, lane 4), addition of eight more residues to the 2aΔC C terminus significantly reduces activity (50). Similarly, deletion of 1a sequences from 1a2aΔNC demonstrated that, while some small deletions may largely abolish function, larger overlapping deletions can be strikingly more active (Fig. 4).

Second, unlike the experiments described here using DNA-based expression of BMV proteins, the prior deletion tests (50) relied on expression of 2a variants from replication-dependent RNA2 in vitro transcripts. This is significant because the first 100 to 200 codons of the 2a gene also contain *cis*-acting replication signals necessary for RNA2 replication (50). Independently of any effect on 2a protein function, deletions in this region interfere with RNA2 replication and thus 2a expression. As first shown by Rao and Hall (38) and Dinant et al. (12), expressing even wt 2a by transfection of a nonreplicating transcript inhibits RNA3 replication at least 10-fold relative to RNA3 levels for wt BMV infection or expression of wt 1a and 2a from the plasmids used in this study. This 10-fold *cis*-acting reduction, perhaps in combination with other effects, may have further obscured detection of activity in the N-terminal 2a deletion mutants tested in this prior RNA2-based study.

Finally, as noted below, the stable, DNA-based expression of 1a and 2a used here may relax some constraints on 1a and 2a function that apply to RNA replication when 1a and 2a are expressed from replication-dependent RNAs.

Possible functions of the N-terminal 2a-1a interaction. Biochemical (24), genetic (49, 50), and phylogenetic (5, 27, 39) considerations suggest that the N-terminal region of wt 2a and its ability to interact with 1a significantly contribute to BMV survival. However, the activity of 2aΔNC in RNA replication showed that the N-terminal region was not essential for RNA3 replication in protoplasts. Among other possible explanations for this apparent dichotomy, the N-terminal 2a-1a interaction might further stabilize the essential interaction between the 2a core and 1a revealed in this study. In natural BMV infection, where 2a is expressed from replicating RNA2 and initial 2a concentrations are low, such enhanced affinity of 2a for 1a may be essential to expedite assembly of the first replication complexes and rescue all three genomic RNAs into replication before the inoculum RNA templates are degraded.

Alternatively, the N-terminal 2a-1a interaction might have a function that is not assayed in the protoplast experiments used here but which is important for BMV survival in whole-plant infection. The C terminus of 2a, e.g., is dispensable for RNA replication in protoplasts, but loss of this region greatly inhibits spread and/or accumulation of BMV in both inoculated and systemically infected tissues of whole plants (50). Whether this effect on infection spread is related to or independent from the role of 2a in RNA replication is presently unclear (10, 11). In addition, the present experiments do not rule out the possibility that the N-terminal region of 2a, though not required for RNA3 replication and transcription, is somehow required for replication of RNA1 or RNA2.

Third, and not mutually exclusive with the above, the N-terminal 2a-1a interaction may provide a selective advantage that becomes apparent only in competition experiments between mutant and wt genomes. For instance, CCMV RNA3 mutants with deletions in either of two large noncoding regions replicate to normal titers in single infections but are quickly outcompeted by wt CCMV RNA3 in coinoculations (35).

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